JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2010, p. 3263–3266 Vol. 48, No. 9
0095-1137/10/$12.00 doi:10.1128/JCM.00819-10

Prevalence, Genetic Characteristics, and Zoonotic Potential of *Cryptosporidium* Species Causing Infections in Farm Rabbits in China▽

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Received 23 April 2010/Returned for modification 14 June 2010/Accepted 28 June 2010

To assess the prevalence and public health significance of rabbit cryptosporidiosis, a total of 1,081 fecal specimens were collected between October 2007 and April 2008 from rabbits on eight farms in five different areas in Henan Province, China, and were examined by microscopy after Sheather's sucrose flotation and modified acid-fast staining. The average infection rate of *Cryptosporidium* was 3.4% (37/1,081 samples). There was a significant association between the prevalence of *Cryptosporidium* and the age of animals ($\chi^2 = 57.13$; $P < 0.01$); the prevalence of cryptosporidiosis in 1- to 3-month-old rabbits was the highest (10.9%). The *Cryptosporidium* species in microscopy-positive specimens were genotyped by sequence analyses of the 18S rRNA, 70-kDa heat shock protein (HSP70), oocyst wall protein (COWP), and actin genes and were subtyped by sequence analysis of the 60-kDa glycoprotein (gp60) gene. Only the *Cryptosporidium* rabbit genotype was identified, with 100% sequence identity to published sequences of the 18S rRNA, HSP70, COWP, and actin genes, and the strains belonged to three gp60 subtypes (VbA36, VbA35, and VbA29). In view of the recent finding of the *Cryptosporidium* rabbit genotype in human outbreak and sporadic cases, the role of rabbits in the transmission of human cryptosporidiosis should be reassessed.

Cryptosporidium spp. are important zoonotic protozoa, infecting at least 260 species of vertebrate hosts, including humans (5). Cryptosporidiosis can be acquired by direct contact with infected individuals or animals or contaminated fomites or by ingestion of contaminated food and water. At present, there are at least 20 established species and more than 60 genotypes of uncertain species status (5, 11, 25, 26). Among these, at least eight species (*Cryptosporidium hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C. muris*, *C. suis*, and *C. andersoni*) and six genotypes (cervine, horse, rabbit, and skunk genotypes, chipmunk genotype I, and pig genotype II) have been detected in immunocompromised and immunocompetent persons, some of which involved only a few cases (3, 5, 6, 12, 27). Since the first description of *Cryptosporidium* in feces of rabbits by Tyzzer in 1912, the organism has been reported for farmed, pet, laboratory, and wild rabbits (7, 9, 10, 13–16, 28).

Cryptosporidium oocysts of rabbit origin are morphologically similar to those found in other animals and humans. The identification of *Cryptosporidium* spp. in rabbits is not well established. Thus, the parasites detected in rabbits have traditionally been named *C. parvum*. Lately, characterizations of the 18S rRNA, 70-kDa heat shock protein (HSP70), oocyst wall protein (COWP), actin, and 60-kDa glycoprotein (gp60) genes of a few isolates from rabbits have led to the identification of the *Cryptosporidium* rabbit genotype (2, 4, 13, 14, 24). Recently, the *Cryptosporidium* rabbit genotype was reported as a pathogen in a few human cases and one outbreak of waterborne cryptosporidiosis in the United Kingdom (4, 12). Thus, rabbit cryptosporidiosis may pose potential risks to public health.

In China, one study reported the prevalence of *Cryptosporidium* in rabbits in Changchun (28), and one characterized two rabbit *Cryptosporidium* isolates at the genotype level (24). The present study was conducted to determine the prevalence and zoonotic potential of *Cryptosporidium* in rabbits in Henan Province, China.

MATERIALS AND METHODS

Sample collection and *Cryptosporidium* oocyst detection. A total of 1,081 fresh fecal specimens were collected from eight rabbit farms in five different areas (Jiyuan, Puyang, Jiaozuo, Zhengzhou, and Nanyang) of Henan Province, China, during October 2007 to April 2008. On each farm, specimens were collected randomly from 15% of the rabbits, with the breed, age, and clinical signs recorded. *Cryptosporidium* oocysts in 5-g specimens were concentrated by Sheather's sucrose flotation and detected by light microscopy of the concentrated materials, which were stained with modified acid-fast stain. The size of oocysts was measured using a Nikon light microscope (Eclipse E200) at a magnification of ×1,000.

DNA extraction. Genomic DNAs were extracted from purified *Cryptosporidium* oocysts of all 37 microscopy-positive specimens by use of a Mag Extractor

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▽ Published ahead of print on 7 July 2010.

TABLE 1. Prevalence of *Cryptosporidium* in rabbits on eight farms in Henan Province, China

Farm	Region	Prevalence of *Cryptosporidium* (no. of positive samples/total no. of samples [%]) in rabbits					Prevalence (%)
		<1 mo	1–3 mo	4–6 mo	7–12 mo	>12 mo	
1	Jiyuan	0/16	0/24	0/62	0/18		0
2	Jiyuan	0/10	14/79	3/58	1/85		7.8
3	Puyang			1/144	0/61		0.5
4	Puyang	0/21		0/106			0
5	Jiaozuo			0/40	0/21	0/30	0
6	Zhengzhou	0/2	0/81			0/27	0
7	Zhengzhou	0/4	0/13	0/49	0/45		0
8	Nanyang	3/20	13/50	2/15			25.7
Total		3/73 (4.1)	27/247 (10.9)	6/474 (1.3)	1/230 (0.4)	0/57 (0)	3.4

genome kit (Toyobo Co. Ltd., Osaka, Japan) according to the manufacturer-recommended procedures. DNAs was kept at −20°C before being used for molecular analysis.

***Cryptosporidium* genotyping at the 18S rRNA, HSP70, COWP, and actin loci.** The 18S rRNA gene of *Cryptosporidium* in microscopy-positive specimens was amplified by nested PCR according to previously described procedures (21). Positive and negative controls were included in each analysis. The PCR products were examined by 1% agarose gel electrophoresis and visualized after GoldView (Guangzhou Geneshun Biotech Ltd., Guangzhou, China) staining. The positive PCR products were analyzed by restriction fragment length polymorphism (RFLP) analysis, using the SspI and VspI enzymes (21, 23). Two-directional sequencing of 16 positive specimens from representative farms, breeds, and age groups was done by TaKaRa Co. Ltd. (Dalian, China) on an ABI Prism 3730 DNA analyzer (Applied Biosystems, Foster City, CA), using a Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems).

Eight isolates from representative farms, breeds, and age groups were selected for further sequence characterization at the HSP70, COWP, and actin loci. Primers and procedures used were adopted from previous studies (17, 18, 22). PCR products of the HSP70 and COWP genes were sequenced as described above, whereas those of the actin gene were sequenced after being cloned into the pGEM1-T Easy vector (Promega).

***Cryptosporidium* subtyping at the gp60 locus.** Specimens were subtyped by amplifying and sequencing the gp60 gene, using primers and procedures described by Alves et al. (1). The subtypes of the *Cryptosporidium* rabbit genotype were named using the established gp60 subtype nomenclature (4, 19), starting with the subtype family designation (Va or Vb for the rabbit genotype) and followed by the specification of the number of TCA repeats (A29, A35, and A36 for the presence of 29, 35, and 36 copies of the TCA repeat).

DNA sequence analysis. Nucleotide sequences obtained from the study were aligned with each other and with reference sequences downloaded from GenBank by use of ClustalX 1.83 (20).

Statistical analysis. Chi-square analysis was performed to analyze the correlation between the prevalence of *Cryptosporidium* and the breed and age of animals, using SPSS 11.5 (Statistical Package for the Social Sciences) for Windows (SPSS Inc., Chicago, IL).

Nucleotide sequence accession numbers. DNA sequences of the partial 18S rRNA, HSP70, actin, COWP, and gp60 genes obtained were deposited in the GenBank database under accession numbers GU097631 to GU097651, HM125714, and HM125715.

RESULTS

Prevalence of *Cryptosporidium* in rabbits. *Cryptosporidium* oocysts were detected by microscopy in specimens from 3 of the 8 rabbit farms in five areas studied. The overall prevalence was 3.4% (37/1,081 samples). Farm 8, in Nanyang, had the highest infection rate (25.7%), followed by farm 2 (7.8%), in Jiyuan, and farm 3 (0.5%), in Puyang. The prevalence rates of *Cryptosporidium* in rabbits aged <1, 1 to 3, 4 to 6, 7 to 12, and >12 months were 4.1%, 10.9%, 1.3%, 0.4%, and 0%, respectively (Table 1). The differences in *Cryptosporidium* prevalence among the age groups were significant ($\chi^2 = 57.13$; $P < 0.01$).

No apparent clinical signs of cryptosporidiosis were seen in rabbits at the time of specimen collection.

***Cryptosporidium* genotypes and subtypes.** PCR amplification of the 18S rRNA gene was successful for 36 of the 37 specimens. RFLP analysis of all PCR-positive products produced identical banding patterns indicative of the presence of the *Cryptosporidium* rabbit genotype: there were three visible bands, of 473, 269, and 109 bp, after SspI restriction and three visible bands, of 559, 104 to 115, and 71 bp, after VspI restriction. DNA sequencing of the 18S rRNA gene PCR products showed that all the sequences obtained in this study were identical to each other and to those previously obtained for the *Cryptosporidium* rabbit genotype, available under GenBank accession numbers AY120901 (24), AY273771 (14), EU437413 (12), and FJ262724 to FJ262726 (4).

PCR products of the COWP gene were sequenced successfully for all eight isolates selected. The sequences obtained were identical to each other and to the one obtained previously for the *Cryptosporidium* rabbit genotype (GenBank accession number EU437411). Likewise, the sequences of the actin gene were also identical to each other and to the one obtained previously for the *Cryptosporidium* rabbit genotype (AY120924) and had a 99.9% similarity to those of *C. hominis* (AF382337, EF591783, and EF591784), with a C-to-T substitution within the 1,066 bp under analysis.

The sequences of the HSP70 gene were nearly identical to each other. Most of the sequences obtained were missing the 3′-end sequence. Among the three sequences with a 3′-end sequence, two had 9 copies of a 12-bp minisatellite sequence commonly seen in the HSP70 gene of *Cryptosporidium* spp. and one had 10 copies of the minisatellite sequences. There was no single nucleotide polymorphism in the HSP70 gene among the *Cryptosporidium* rabbit genotype specimens, and the sequences obtained in this study were identical to the partial sequences (GenBank accession numbers AY273775, EU437412, and FJ262727 to FJ262729; all with the 3′ end missing) obtained previously for the *Cryptosporidium* rabbit genotype. Depending on the *C. hominis* sequence used as a reference, there were 11 to 13 nucleotide substitutions between *C. hominis* and the rabbit genotype, with 7 or 8 of them occurring in the minisatellite region and 4 or 5 of them occurring in the rest of the gene.

Subtypes of the *Cryptosporidium* rabbit genotype. Sequences of the gp60 gene were obtained successfully for 30 of the 37

TABLE 2. Distribution of gp60 subtypes of the *Cryptosporidium* rabbit genotype in rabbits in this study

Farm	Breed	Age (mo) (*n*)	Isolates	No. of samples with gp60 subtype		
				Vb29	Vb35	Vb36
2	Standard Rex	1–3 (10), 4–6 (4), 7–9 (1)	JY1 to JY15	3	4	8
8	Standard Rex	<1 (1), 1–3 (7), 4–6 (1)	NY2 to NY4, NY6, NY8, NY12 to NY15	9	0	0
	New Zealand White	<1 (1), 1–3 (5)	NY1, NY5, NY7, NY9 to NY11	6	0	0

Cryptosporidium rabbit genotype-positive isolates in this study. Three subtypes belonging to one subtype family were identified and were designated VbA29 (in 18 specimens), VbA35 (in 4 specimens), and VbA36 (in 8 specimens). Farm 8 had only VbA29, whereas farm 2 had all three subtypes (Table 2).

Morphometrics of *Cryptosporidium* oocysts. The size of *Cryptosporidium* rabbit genotype oocysts obtained in this study was 4.9 to 5.4 by 4.5 to 5.1 μm (mean ± standard deviation [SD] = 5.1 ± 0.11 by 4.8 ± 0.15 μm; $n = 50$), with a shape index of 1.02 to 1.13 (mean ± SD = 1.07 ± 0.03), similar to the size of *C. hominis* oocysts (mean = 5.2 by 4.9 μm; range = 4.4 to 5.9 by 4.4 to 5.4 μm) (8).

DISCUSSION

An overall 3.4% prevalence (37/1,081 samples) of cryptosporidiosis was seen in domestic rabbits. This is higher than rates reported for wild rabbits in previous studies but at the lower end of rates reported for farmed, laboratory, and pet rabbits (13). Differences in techniques used for the detection of infection and in the management and age of animals probably contributed to the observed variations in the prevalence of cryptosporidiosis in rabbits. In the present study, *Cryptosporidium* oocysts were detected in many rabbits on two farms (farms 2 and 8), and animals of 1 to 3 months of age had much higher infection rates than those in other age groups (Table 1).

In this study, only the *Cryptosporidium* rabbit genotype was identified in rabbits. Sequence similarity analysis revealed that the 18S rRNA, actin, and COWP gene sequences obtained in this study were identical to each other at each locus and had 100% identity to the *Cryptosporidium* rabbit genotype isolates from rabbits and humans found in previous studies (4, 12, 14, 24); only sequences of the HSP70 gene had minor differences, which were seen in the copy number of a minisatellite repeat. Prior to this study, only five publications, representing four studies, characterized *Cryptosporidium* spp. in rabbits. Thus, only a single nucleotide sequence each is available for the actin and COWP loci, and five very short sequences (<400 bp) are available for the HSP70 locus (13). The *Cryptosporidium* rabbit genotype has nucleotide sequences very similar to those of *C. hominis* at the 18S rRNA (only four nucleotide substitutions compared to sequence AJ849462 [within a 787-bp region]), COWP (no substitution compared to sequence DQ388389 [within a 535-bp region]), and actin (only one nucleotide substitution compared to sequence EF591784 [within a 1,066-bp region]) loci. More nucleotide substitutions were seen in the HSP70 gene, especially at the 3′ end of the sequence (11 to 13 substitutions in the 1,920-bp sequence obtained in this study). At this locus, *C. hominis* has 12 or 13 copies of the 12-bp minisatellite sequence, whereas the rabbit genotype has only 9 or 10 copies. There is a need for further studies of the taxonomic relationship between *C. hominis* and the *Cryptosporidium* rabbit genotype to establish the validity of the name *Cryptosporidium cuniculus*, previously used to describe the parasite in rabbits.

Sequence analysis of the gp60 gene is useful for investigating transmission dynamics, for identification and differentiation of outbreaks, and for tracking infection sources (23). In recent studies, several *Cryptosporidium* rabbit genotype isolates from humans and rabbits were subtyped at the gp60 locus. Thus far, two subtype families have been identified, including the Va subtype family, in humans (VaA18 and VaA22) and rabbits (VaA18) in the United Kingdom, and the Vb subtype family, in rabbits in the Czech Republic (VbA19) and China (VbA29) (4). In this study, the gp60 sequence analysis revealed that the isolates of this study belonged to three subtypes (VbA29, VbA35, and VbA36) in the Vb subtype family, which has thus far not been seen in humans.

In conclusion, the *Cryptosporidium* rabbit genotype is a common parasite infecting farmed rabbits in Henan, China. Although there has not been any reported human infection with the parasite in China, its genetic similarity to *C. hominis* and the recent finding of the parasite in humans in the United Kingdom indicate that rabbits can be a potential reservoir of zoonotic cryptosporidiosis. More systematic biologic characterizations of the parasite are needed to understand the taxonomic status of the *Cryptosporidium* rabbit genotype and its public health significance.

ACKNOWLEDGMENTS

This study was supported in part by the National Natural Science Foundation of China (grants 30771881, 30871863, and 30928019), the Ph.D. Programs Foundation of the Ministry of Education of China (grant 20094105110003), the State Key Laboratory of Veterinary Etiological Biology, the Lanzhou Veterinary Research Institute, the Chinese Academy of Agricultural Sciences, and Ministry of Health special funds for public sector research (grant 200808012).

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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